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Molting gland in *Gammarus fossarum* C.L. Koch, 1836: characterization, histogenesis and *in vitro* ecdysteroids biosynthesis

Линочная железа у *Gammarus fossarum* C.L. Koch, 1836: характеристика, гистогенез и биосинтез экдистероидов *in vitro*

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КЛЮЧЕВЫЕ СЛОВА: Y-орган, гистология, экдистероиды, гистогенез, *Gammarus fossarum*.

ABSTRACT. The anatomy of the molting gland (Y-organ) in fresh water amphipod *Gammarus fossarum* as well as embryological and histological details of this gland are described. Y-organ is located on both sides of the oral cavity, at the level of the maxilla and mandible insertion. Its surface varied from 3100 to 4050 μm^2. It is composed of lobules; each lobule contains 6 to 13 basophilic cells. In post molt, Y-organ appeared compact with an average area of 3200±48 μm^2. During premolt Y-organ becomes larger (3800±204 μm^2) and produces a high level of ecdysteroids. In embryos, Y-organ appears during third stage. In the 4th stage, the gland is wide. Its cells are more enriched in secretory granules and its nuclear size increases (ANOVA, p-value 0.0002). During stage 5, we observed a significant decrease in area of the gland (ANOVA, p-value = 0.05). A positive correlation (r^2 = 0.88) was observed during the development stages between gland surface and size of its cells.

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РЕЗЮМЕ. Описана анатомия линочной железы (Y-органа) пресноводного бокоплава *Gammarus fossarum*, также приведены эмбриологические и гистологические данные. Y-орган расположен по обеим сторонам ротовой полости, на уровне присоединения максилл и мандибул. Площадь его поверхности варьирукет от 3100 до 4050 мкм^2. Он состоит из лопастей; каждая лопасть содержит от 6 до 13 базофильные клетки. В послелиночный период, Y-орган становится компактным с площадью 3200±48 мкм^2. В предлиночный период Y-орган становится больше (3800±204 мкм^2) и продуцирует много экдистероидов. В эмбрионе Y-орган появляется на третьей стадии. На 4-й стадии железа становится широкой. Ее клетки обогащаются секреторными гранулами, размер их ядер возрастает (ANOVA, p-значение 0,0002). На 5-й стадии наблюдается значительное уменьшение объема железы (ANOVA, p-значение = 0,05). Во время развития выявлена позитивная корреляция (r^2 = 0.88) между поверхностью железы и размером ее клеток.

Introduction

Gammarus fossarum C.L. Koch, 1836is an important part of the aquatic macroinvertebrate assemblage [Dedourge-Geffard *et al*., 2009; Ladewig *et al*., 2006]. It has been used as a model species due to its wide distribution in Europe [Janetzky, 1994] and its major functional role in litter breakdown process and nutrient cycling [MacNeil *et al*., 1997; Forrow, Maltby, 2000; Kelly *et al*., 2002; Lacaze *et al*., 2010]. *G. fossarum* is used as sentinel species in freshwater risk assessment, for several reasons [Kunz *et al*., 2010]. First, it occupies a large trophic repertoire: herbivores, predators, and detritivores playing a major role in leaf-litter breakdown processes. Second it's widespread and found throughout a large habitat range, where it often occurs at high densities [Dangles, Guerold, 2001]. It constitutes a food reserve for macro invertebrates and fish. Finally, gammarids can be easily maintained in the

laboratory or used for in situ bioassays [Kunz *et al*., 2010].

With this specie we can assess the impact of pollutants by measuring molecular markers related to diverse modes of action, such as neurotoxicity [Xuereb *et al.*, 2009], as well as by using life-history-trait reproductive features [Geffard *et al.*, 2010]. Alterations of sexual phenotype (intersexuality) have been reported in situ. Jungmann *et al*. [2004], as well as alterations by xenobiotics of various physiological parameters related to reproductive success (i.e. gametogenesis, embryogenesis, fecundity, or molt) [Geffard *et al.*, 2010]. However, the mechanisms involved in these reproductive impairments are unknown. A major reason for this is that molting gland (Y-organ) involved in the regulation of reproductive function in Gammarid in general, have until recently been largely misunderstood [LeBlanc, 2007]: although *Gammarus* is a relevant ecotoxicological animal model, information about Y-organ are lacking for this genus. According to the literature, the Y-organ was identified in *Carcinus maenas* Linnaeus, 1758 [Gabe, 1956], *Orchestia gammarella* (Pallas, 1766) [Blanchet, 1974], *Penaeus indicus* (H. Milne Edwards, 1837) [Vijayan *et al*., 2003] *Metapenaeus* sp. [Dall, 1965], *Palaemon paucidens* De Haan, 1844 [Auto *et al*., 1974], *Penaeus japonicus* Spence Bate, 1888 [Bourguet *et al* ., 1977], *Metopograpsus messor* (Forskal, 1775), *Palaemon serratus* (Pennant, 1777) [Le Roux , 1977], *Astacus astacus* Linnaeus, 1758 [Birkenbeil, Gersch, 1979], *Metapenaeus* sp. [Shyamal *et al*., 2014], *Orchestia cavimana* Heller, 1865 [Graf, Delbecque, 1987]. Subsequent investigations involving organ culture experiments have identified the crustacean molting hormone as ecdysteroids, belonging to polyhydroxylated C27 steroids, and 20-hydroxyecdysone as crustecdysone [beta ecdysone], the major molt-stimulatory principle [Bollenbacker, O'Connor, 1973; Chang *et al*., 1993]. Further, it became evident that the Y-organ secretes ecdysone, which in turn is converted into 20-hydroxyecdysone [Chang, O'Connor, 1977; Lachaise *et al*., 1989].

Ecdysteroid is regulated at the level of synthesis and secretion by a neuropeptide hormone namely molt inhibiting hormone present in the eyestalk X-organsinus gland complex. Eyestalk ablation causes an increase in ecdysteroid secretion, suggesting that there are inhibitory factors in the eyestalk that suppress ecdysteroid secretion.

Later research has unraveled the mechanism of steroid uptake through energy-requiring process involving Na/K-ATPase [Spindler, O'Connor, 1980]. Investigations con ducted at molecular levels during the 1990's and the 2000's have thrown light on the receptor-mediated mechanism of [molt] hormone action [Hopkins, 2009].

In the fresh water *G. fossarum* molts and ovarian maturation occurs simultaneously, with the full cycle of ovarian development; here, we have documented gammarid endocrine system through the identification and characterization of Y organ in adult and embryos . For this, we investigated the structure and histogenesis of Y-organ in *G. fossarum*. We have considered stage B [post molt] and stage D1 (premolt) to assess the Y-organ secretory activity and the fluctuating ecdysteroids levels in relation to molt and/or reproduction of females. For embryos we have considered all development stages, in order to identify exactly the stage of Y-organ apparition. Results were used in ecotoxicological research by describing the mechanism of action of chemicals on Y-organ anatomy and physiology [Abidi *et al*., 2016].

Material and methods

Collection and maintenance of *Gammarus fossarum*

Animals recovered were collected using a net (by kick sampling) from La Tour du Pin, upstream of the Bourbre River (eastern central France). This site has good water quality according to RNB data records (Reseau National de Bassin, the French Watershed Biomonitoring Network; http://sierm.eaurmc.fr/eaux-superficielles/index.php). Immediately after sampling, specimens were quickly transferred to the laboratory. Organisms were kept during an acclimatization period of 10 to 15 d, in 30-L tanks continuously supplied with drilled groundwater adjusted to the sampling site conductivity (i.e., 600 $mS.cm^{-1}$) and under constant aeration. The temperature was kept at 12±1 °C and a 16/8 h light: dark photoperiod was maintained. Organisms were fed ad libitum with alder leaves (*Alnus glutinosa*). The leaves were conditioned for at least 6±1 d in water. Free zedried *Tubifex worms* (Europrix) were provided as a dietary supplement twice a week.

Identification of pre- and postmolt stages in females and development stages in embryos

Post and premolt stages of *G. fossarum* females were characterized and were identified using criteria developed by Geffard *et al*. [2010] the first and second periopod pairs (dactylopodite and protopodite) of females were mounted on a microscope slide with a coverslip, and their integumental morphogenesis was observed. The post molt stage (Stage B) is characterized by the hardening of the cuticle and is easily observed to the dactylopodite setae level. On premolt satge(stage D1) we observed cuticle on the new dactylopodite. Development stage of the embryos and their occurrence were defined using the criteria proposed by Geffard *et al*. [2010]. Embryos were manually recovered from the marsupium, placed on a slide with water (water used for acclimatization), and morphological changes were observed under the stereo microscope. On Stage 1 embryos were observed, corresponding to newly fertilized, oval, and undifferentiated eggs. On stage 2 embryos were characterized by a comma-like shape. Stage 3 was characterized by the presence of cephalothorax and segmented appendages. At the next

stage 4, the compound eye was fully visible and appendages were also fully developed. The last stage 5 corresponds to newly hatched juveniles.

Histology

Embryos (5 for each development stage) and adult cephalons (stage B (5samples); D1 (5 samples) were placed in Bouin's fluid for 48 h, rinsed and dehydrated in a graded series of ethanol. Thereafter, samples were included in Paraplast X-tra1 (Sigma-Aldrich). Serial 5-µm (for cephalon) and 1 µm (for embryos) cross and longitudinal sections were then made and colored using hemalum-eosin (Sigma-Aldrich) [Martoja, Martoja-Pierson, 1967].

Slides were observed with a Leica DM 25001 microscopes. Measurements (with Mesurim logiciel)of whole organ and nuclei surface were carried out for each stage (B and D1). Similar approach was performed in embryos but with measurement of the cell surface. All surface (µm^2) measurements were made on microscopic magnification x100 using the Nis-Elements D imaging software version 4.12.00. For Y-gland females and embryos, the number of replicas was = 5 while the number of cells and nuclei which measured the surface was = 20.

***In vitro* culture of Y-organ**

We relied up on our histological localization of Y organ for the localization of the tissue. After having dissected out (by cutting open the cephalon), the gland was immediately put in 200 µl of medium culture (M199 + fetal serum calf (10%) + antibiotics: streptomycinn and penicillin (adjusted to the *G. fossarum* osmolarity). Five Y-organs were incubated in each well of 96 well microplate with of medium culture. Incubation temperature was 16 °C during 72 hours 2.5.

Ecdysteroid level

The produced ecdysteroids released by Y-organ was measured using the EIA (Enzymimmunoassay) procedure described by Maniere *et al.* [2004] and adapted to gammarids in the UMR 5023 from the University of Lyon 1. This method is based on competition between ecdysteroids and a tracer (2succinyl-20-hydroxyecdysone bound to peroxidase) for anti-ecdysteroids antibodies. A L2 Polyclonal detects many ecdysteroids, ecdysone mainly and 2-Deoxy-E, but 6 times less sensitive to the 20-hydroxyecdysone (20^E).Each analyzed sample was measured at least in duplicate. 20he was used for references curves, ecdysteroids titers are expressed in 20HE equivalents [Mondy, Corio Cotest, 2000].

Statistical studies

For assessing the statistical significance in the fluctuation of Y-organ surface during molting stage in female and development stage in embryos, the data were subjected to Student's paired t Test. Other means were compared by one-way ANOVA (Tukey-test). All values are expressed as means± deviation. Statistical procedures were carried out with the Statistica 9.

Results

Localization and histological study of Y-organ in females of *Gammarus fossarum*

Y-organ is located on both sides of the oral cavity, next to circumoesophageal connective (COC) and at the level of the muscle insertion of the maxillule and mandible (Fig. 1A). A part of this organ is inside the body, while the other protrudes is in the ventral portion and forms a cuticular swelling. The internal cuticle of this region has an average height of 33 ± 0.7 µm (Fig. 1B). Area of Y-organ in *G. fossarum* varies from 3100 to 4050 µm^2. Morphologically, the gland contains lobules; in each lobule we observe 6 to 13 basophilic cells (affinity for haematoxylin). Most cells are fusiform with clear cytoplasm. Membranes and cell boundaries are barely visible. The nucleolus is oval or of spherical shape with a dense chromatin (Fig. 1B).

Molting Stage-dependent fluctuation of Y-organ in female *Gammarus fossarum*

Our observation noted that morphology and size of Y-organ exhibited molting-dependent changes. In post molt when female was not engaging either in reproduction Geffard *et al*. [2010], the Y-organ appeared compact with an average area of 3200±48 µm^2. The cytoplasm is clear and slightly rich in granules. The average surface of the nuclei is 30.8±4 µm^2. During premolt, the gland, appeared significantly larger (3800±204 µm^2, ANOVA p-value: 0.0003) (Table 1). Concerning nuclei surface, there is a significant difference between premolt and post molt (p-value: 0.002) (Fig. 2).

Quantification of ecdysteroids secreted by Y-Organ in vitro and in the hemolymph of female (stage B and D1)

Table 2 showed ecdysteroids production by Y-organ after 72 hours of incubation. We confirm that female in post molt produces less quantity of ecdysteroids than female in premolt stage.

Y-organ in embryo *Gammarus fossarum*

During the first stage of embryonic development, we undifferentiated cells. In stage 2, the retroflex appeared at the level of the egg and a strong cellular differentiation was observed in maxillar zone: primitive epidermal cubicular cells of the molting gland appeared. These cells are distinguished by their large cytoplasm and a dense nucleus pushed to the side of the cell. During third stage, we note that structure of Y-organ becomes clear. Glandular cells keep a direct insertion to the cuticle: a bulge of the gland located between the maxilla and the mandibule; it takes an arc form delimited and separated from other structures.

Microscopic observations of Y organ showed that in stage 4, the gland becomes wide, cells are more

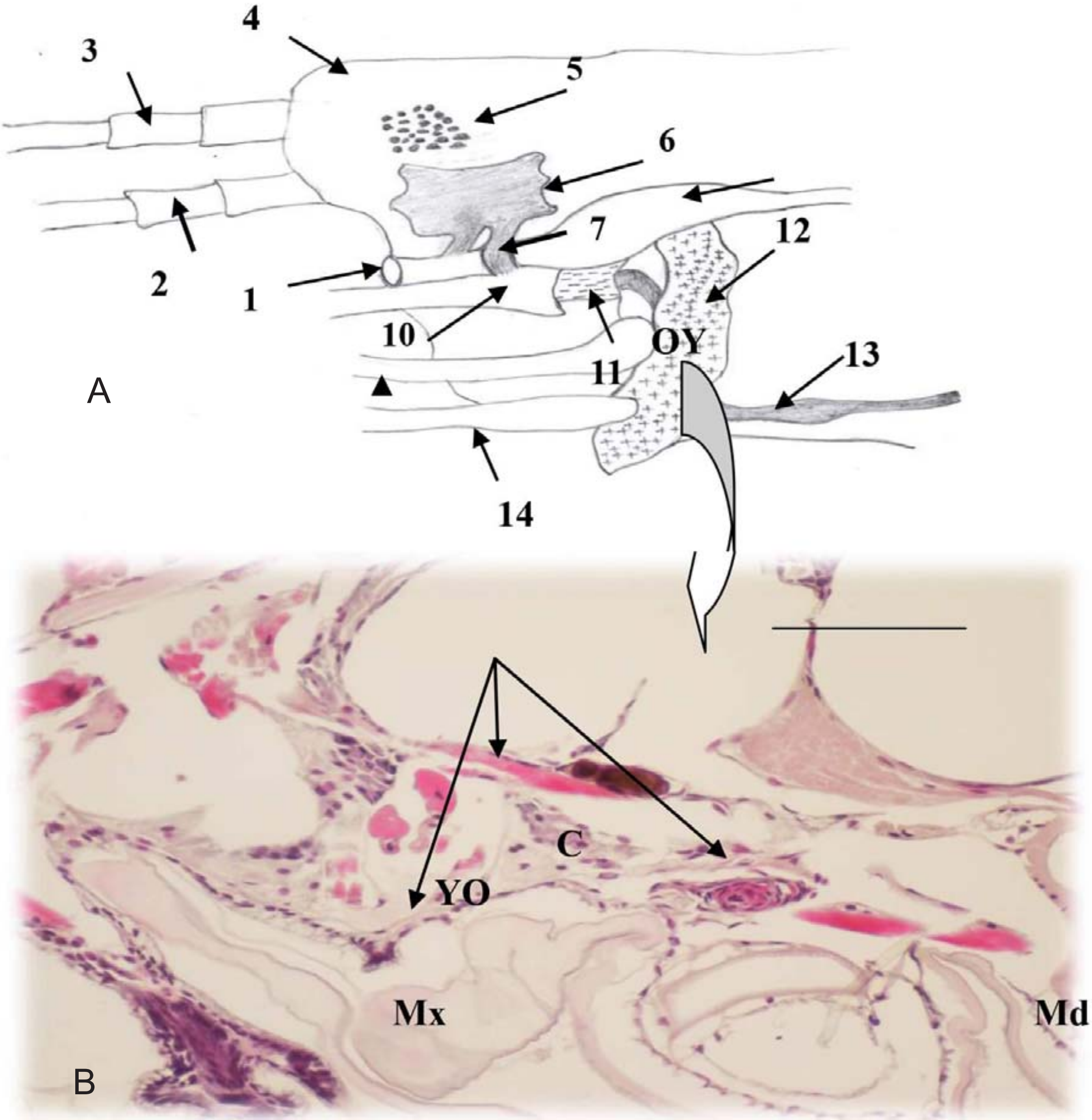


Fig. 1. A — cephalon of *Gammarus fossarum* schematized from observations of serial longitudinal and transversal sections: 1 — mouth (anterior part); 2 — antenna 2; 3 —antenna 1; 4 — head; 5 — ommatidia; 6 — OX/GS complex; 7 — nerveous collar; 8 — mouth (posterior part); 9 — mandibule; 10 — mandibular organ; 11 — Y-organ; 12 — nervous cord; 13 — maxilla 2; 14 — maxilla 1. B — localization of Y-organ in cephalon of *G. fossarum*: Y-organ (O) at the level of a cuticular swelling (C.S) (33.06 ± 0.66 µm in height) between the maxilla (M) and the mandible (Md). Molting gland is formed by lobules, each of them consist of 6 to 13 cells; C — cuticle (scale bar: 20 µm; longitudinal section).

Рис. 1. A — цефалон *Gammarus fossarum*, схематизирован по сериальным продольным и поперечным срезам: 1 — рот (передняя часть); 2 — антенна 2; 3 —антенна 1; 4 — голова; 5 — омматидии; 6 — комплекс OX/GS; 7 — нервный воротничок; 8 — рот (задняя часть); 9 — мандибула; 10 — мандибулярный орган; 11 — Y-орган; 12 — нервная цепочка; 13 — максилла 2; 14 — максилла 1. B — размещение Y-органа в цефалоне *G. fossarum*: Y-орган (O) на уровне кутикулярного изгиба (C.S) (33.06 ± 0.66 мкм в высоту) между максиллой (M) и мандибулой (Md). Линочная железа образована лопастями, каждая из которых состоит из 6–13 клеток; C — кутикула (масштаб 20 мкм; продольный разрез).

Table 1. Area of the molting gland and its nuclei during premolt (Stage B) and post molt (Stage D1).
Таблица 1. Площадь линочной железы и ее ядре в предлиночный (Stage B) и послелиночный период (Stage D1).

	Molting stages			
	Stage B		Stage D1	
	Y-organ	Nuclei	Y-organ	Nuclei
Area (µm^2)	3188*	30,7*	3778*	42*
Standard déviation (St.Dev)	84	4,1	207	10

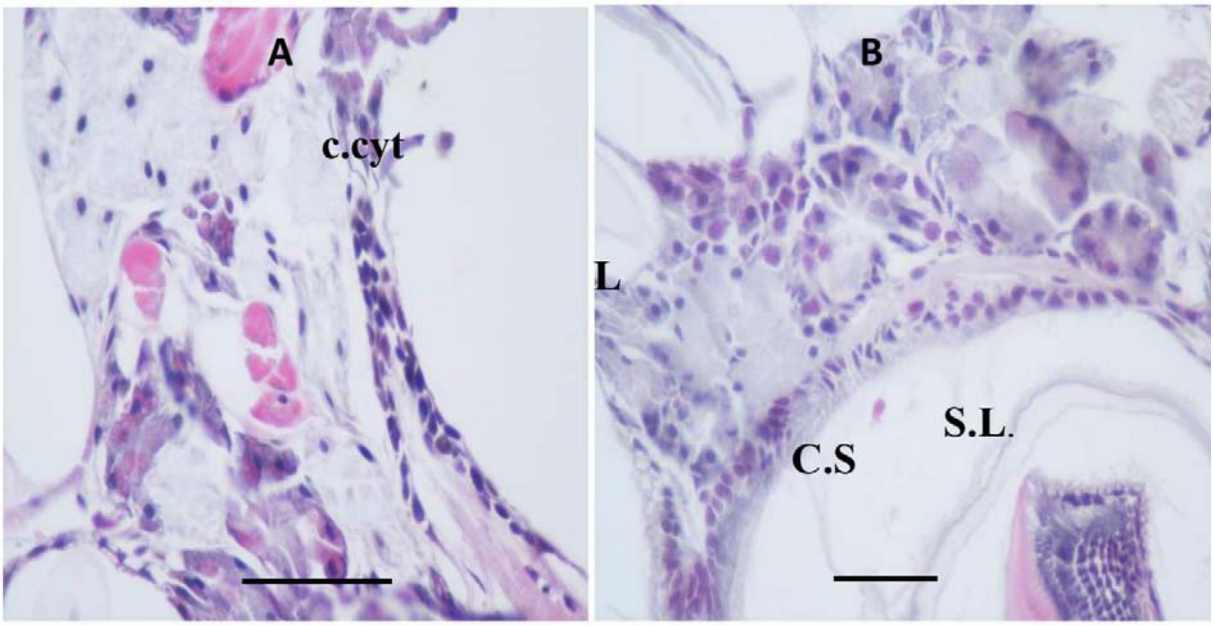


Fig. 2. Y-organ structure of *Gammarus fossarum* females in post molt (A) and premolt (B). In post molt (A) the gland is compact; the cytoplasm is clear, slightly rich in granules. In premolt (B], the gland is wide and more complex: the cellular lobules are superimposed. The nuclei grow (surface: 42,73 ± 10 μm^2) indicating a high metabolic activity that prepare to the molting process. Scale bar: 100 µm. L — lobule; S.L — superimposed lobules; c.cyt — clear cytoplasm.

Рис. 2. Строение Y-органа самки *Gammarus fossarum* в послелиночном (А) и предлиночном (В) состоянии. В послелиночном состоянии (А) железа компактная; цитоплазма чистая, слегка обогащенная гранулами. В предлиночном состоянии (В), железа широкая и более комплексная: клеточные лопасти наложены друг на друга. Рост ядер (поверхность: 42,73 ± 10 мкм^2) показывает высокую метаболическую активность, связанную с подготовкой линьки. Масштаб 100 мкм. L — лопасть; S.L — наложенные лопасти; c.cyt — чистая цитоплазма.

Table 2. Ecdysteroids production by the Y-organ after 72 hours incubation.
Таблица 2. Продукция экдистероидов Y-органом после инкубации в течение 72 ч.

	Molting stages	
	stage B	stage D1
Ecdysteroids/Y organ Ecdysteroids/200 µl pf culture mefim	200	2354

enriched in secretory granules and its nuclear volume increases (Fig. 3) (ANOVA, p-value = 0.0002). During stage 3 and 4, we observed many morphological evolutions: the cephalothorax and the segmentation occur, cephalic and first thoracic appendages of the embryo are then differentiated. Also, the abdomen is folded under the thorax and the telson then locates opposite the cephalic appendages. In stage 5, we observed a significant decrease in the size of the gland and its cells (ANOVA, p-value = 0.05), but it was statistically near to stage 3(ANOVA, p-value = 0.55) (Fig. 3). A positive correlation ($r^2 = 0.88$) was observed during development stages between gland surface and size of its cells (Fig. 4).

Discussion

In order to grow and to increase in size, crustaceans must periodically replace their protective exoskeletons with larger ones by a process called molting, which is under hormonal control. Ecdysial gland (Y-organ) has been widely accepted as the seat of growth stimulation, ever since Gabe [1956], identified a pair of ductless glands in malacostracans, comparable to the one in insects and elucidated the role of these glands in molting of the crab *Carcinus maenas* [Gabe, 1956].

The present paper describe i) localization, morphology, structure and secrotry activity of the Y-organ in the amphipod (*G. fossarum*), ii) the gland's molting

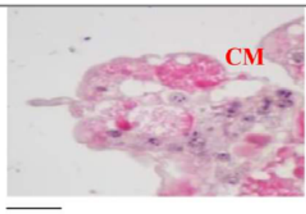
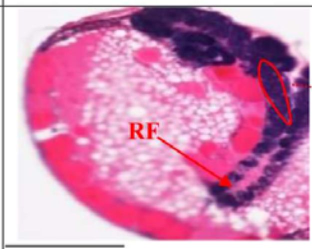
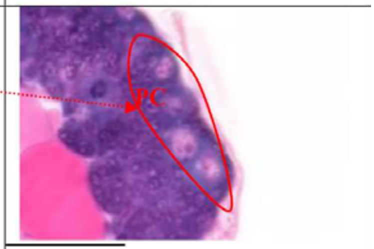
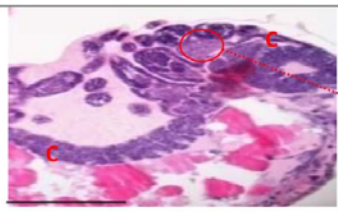
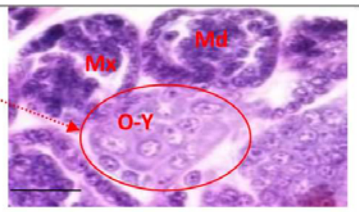
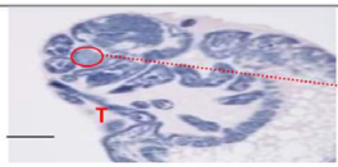
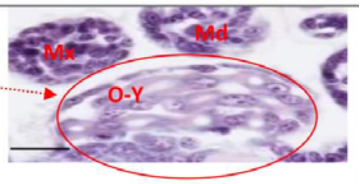
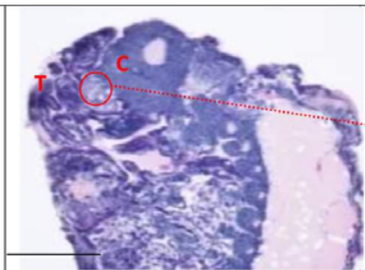
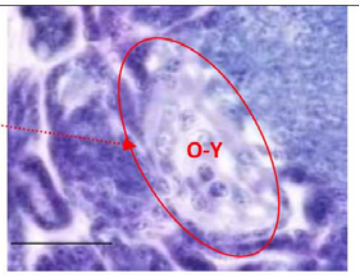
Stages of embryonic development	A- The embryos aspect [Scale bars: 20 µm]	B- Y-organ [Scale bars: 100 µm]
Stage 1		Absent
Stage 2		
Stage 3		
Stage 4		
29 **Stage 5**		

Fig. 3. Histogenesis of the Y-organ in *Gammarus fossarum* embryo: in stage 2, we note appearance of the retroflexion at the level of the egg. Primitive cubic cells of molting gland give an uni-stratified aspect to the gland. At stage 3, there is the cephalothorax and the segmentation of different parts of the body. Y-organ becomes visible; the gland loses its uni-stratified appearance and becomes massive. PC — primitive cells of Y-organ; YO — Y-organ; Md — mandible; Mx — maxilla, CM — cells in mitosis; RF — the retroflexion; T — telson; C — cuticle.

Рис. 3. Гистогенез Y-органа в эмбрионе *Gammarus fossarum*: на 2-й стадии мы наблюдаем появление ретрофлексии на уровне яйца. Примитивные кубические клетки линочной железы придают ей однослойный аспект. На 3-й стадии видна головогрудь и сегментация разных частей тела. Y-орган становится видимым; железа теряет однослойный вид и становится массивной. PC — примитивные клетки Y-органа; YO — Y-орган; Md — мандибула; Mx — максилла, CM — клетки в митозе; RF — ретрофлксия; T — тельсон; C — кутикула.

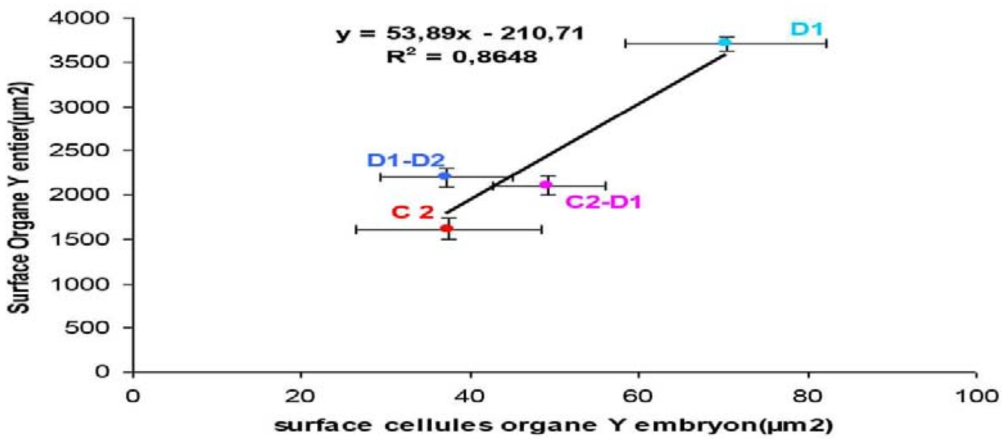


Fig. 4. Positive correlation between the area of Y-organ and the size of its cells in *Gammarus fossarum* during the different stages of molt (C2, C2 – D1, D1, D1 – D2).

Рис. 4. Позитивная корреляция между объемом Y-органа и размером его клеток у *Gammarus fossarum* во время разных стадий линьки (C2, C2 – D1, D1, D1 – D2).

changes at histological 3) histogenesis of the Yorgan in embryos.

Concerning its localization, the Y-organ of *G. fossarum* appears comparable with the "amphipods" type described by Gabe [1956] and Blanchet [1974]. Indeed, it's localized at the level of the cephalon in the second metamere maxillary, and presents a visible local cuticular thickening. In its gross morphology Y-organ, appears comparable with *Penaeus indicus* [Vijayan *et al*., 2003], *Metapenaeus* sp. [Dall, 1965], *Palaemon paucidens* [Auto *et al*., 1974] and *Penaeus japonicus* [Bourguet *et al*., 1977].

Our present study establishes also the existence of perceptible changes in the Y-organ in terms of its morphology and structure, in relation to molting stage (premolt (stage B) and post molt (stage D1). During premolt, we observe an increase in the gland size and it's nucleus. This state was reported in cell of Y-organ of *Metopograpsus messor*, *Palaemon serratus* [Le Roux, 1977] and *Astacus astacus* [Birkenbeil, Gersch, 1979]. According to authors, these morphological changes observed refer a high metabolic activity. Birkenbeil and Gersch [1979] suggested that at this stage the active Golgi apparatus are surrounded by endoplasmic reticulum well developed with a number of ribosomes and are more important than other stages of molting. A comparable situation was found to exist in the freshwater prawn, *Palaemon paucidens*, *Metapenaeus* sp. [Shyamal *et al*., 2014] and the brachyuran crab, *Carcinus maenas* where in the macromitochondria were considered to be involved in elevated synthetic activity.

Superimposing the data obtained from the present histological studies, on our results of ecdysteroid produced *in vitro* by Y-organ in post molt and in premolt, makes it evident that fluctuations in ecdysteroid levels are in consonance with the secretory activity of the Y-organ during molting stage. Indeed, during premolt when Y gland is widen we observe a high amount of ecdysteroids. In post molt, size of Y-organ reduces so the ecdysteroids production decreases. As a result, we suggest a high probably role of the Y organ in molting. During stage D when *G. fossarum* is preparing for ecdysis [Fingerman, 1987] and molting Geffard *et al*., 2010]; the Y organ secrete a high level of ecdysteroids. Immediately following ecdysis, *G.fossarum* was observed with new cuticule [Geffard *et al*., 2010]; The Y-organ activity declines, and ecdysteroids are maintained at basal levels [Abidi *et al*., 2016]. The role of Y organ in the initiation of molting and reproduction also confirmed in *Orchestia cavimana* [Graf, Delbecque, 1987] and *O. gammarella* [Blanchet *et al*., 1979].

Concerning embryos, microscopic observations of histology showed absence of Y-organ at early development stage. Authors [Le Roux, 1977, Sollaud, 1924; Richard, 1974] noted also the difficulty of detecting embryo Y-organ of the sub family of the Palaeraoninae in the early stages of embryonic development. They suggest that there is an appearance of this gland after ontogeny. According to Block *et al*. [2003], development of embryos in this stage depends to maternal ecdysteroids and peak levels (295 pg/mg) occur just prior to molting [Graf, Delbecque, 1987].

In the second stage, when cephalon is present, we noted apparence of epidermal primitive cells with remarkable size. These cells were arranged next to each other so as to provide an unistratified aspect. According to Le Roux [1977] those cells should be the first manifestations and the emergence of the Y organ. At stage three of the embryonic development, Y-organ is present and its structure appears different from surrounding tissues. During stage four, area of the Y-gland and its cells increases significantly This state was, high probably, due to the generation Y-organ. In parallel, we note a high progression in morphogenesis

of embryos: apparition of thoracic appendage, the telson and the juvenile form become apparent (Fig. 3A-stage 4).

Conclusion

Having knowledge on molting stage and embryonic development in *Gammarus fossarum* [Geffard *et al*., 2010] helped us to obtain a clear idea on localization, morphology, histogenesis and secretory activity of Y organ as well as the fluctuation of its morphology in adult during post-molt and premolt. The perceptible difference in the ecdysteroid levels and morphology of the Y-organ, existing between the postmol et and premolt, deserves attention on its possible involvement in molting and reproduction.

We have, also, described a number of aspects of the Y-organ during embryogenesis that make it particularly attractive for developmental and evolutionary studies. The embryos are robust and highly resistant to environmental variations in temperature and salinity and are suitable for a wide range of experimental manipulations. Additionally, the embryos are direct developers and the hatchlings are morphologically similar to adults.

Compliance with ethical standards

CONFLICTS OF INTEREST: The authors declare that they have no conflicts of interest.

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